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BCL2L10 is frequently silenced by promoter hypermethylation in gastric cancer

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Abstract. In gastric cancer, several tumor suppressor and tumor-related genes are silenced by aberrant methylation. Previously, we demonstrated that BCL2L10, which belongs to the pro-apoptotic Bcl-2 family, was transcriptionally repressed by promoter hypermethylation and that its overexpression caused apoptosis and growth inhibition of gastric cancer cells. In this study, we investigated the methylation status of BCL2L10 and its expression in 21 gastric cancer tissues and corresponding non-neoplastic mucosae along with the methylation status of p16, RUNX3, and hMLH1 genes by using methylation specific PCR. In addition, we examined the association between the methylation status of each gene and the expression of EZH2, which was associated with DNA methylation of its target genes. As a result, aberrant methylation of BCL2L10 was detected in 38% of gastric cancer and in 24% of corresponding non-neoplastic mucosae and correlated with low expression of BCL2L10. Methylation of p16, RUNX3, and hMLH1 was found in gastric cancer and in corresponding non-neoplastic mucosae at almost similar frequencies as previous reports. Expression of EZH2 was detected more frequently in tumors (48%) as compared to corresponding non-neoplastic mucosae (10%) (p=0.006), however, no significant difference was found between expression of EZH2 and the methylation frequency of each gene. In conclusion, our data suggest that silencing of BCL2L10 by aberrant methylation is a common feature in gastric cancer and its inactivation may be involved in the early steps of gastric carcinogenesis.

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Introduction

Transcriptional silencing of tumor suppressor genes by promoter hypermethylation is a common feature of human cancer. In gastric cancer, several tumor suppressor and tumor-related genes, including CDKN2A (p16) (1), RUNX3 (2) and hMLH1 (3), have been reported to be silenced by aberrant methylation. Recently, the number of genes known to be inactivated by DNA methylation in gastric cancer, such as genes related to cell cycle control (4), cell proliferation (5) and apoptosis (6), have accumulated. We previously analyzed the genes induced by the demethylating agent 5-aza-2'-deoxycytidine (DAC) in gastric cancer cell lines using a cDNA microarray containing 30,000 genes. We found that BCL2L10, which belongs to the proapoptotic Bcl-2 family, was transcriptionally repressed by promoter hypermethylation (7). BCL2L10 (also called Diva) has been reported to directly interact with Apaf-1 and to displace Bcl-X_L, suggesting it inhibits Bcl-X_L function and promotes apoptosis (8). Conversely, it was also reported that BCL2L10 had an anti-apoptotic effect on human glioma cells (9). The previous observations that BCL2L10 was associated with either the anti-apoptotic proteins Bcl-2 and Bcl-X_L or with the pro-apoptotic protein Bax (10) suggest that BCL2L10 could exert different effects on cells under certain circumstances depending on the cellular context. Previously, we demonstrated that overexpression of BCL2L10 caused apoptosis and growth inhibition of gastric cancer cells (7). Gastric carcinogenesis is thought to consist of a multi-step process composed of genetic and epigenetic disorders. Methylation-mediated down-regulation of BCL2L10 may be one such epigenetic event involved in gastric carcinogenesis.

Recently, it was reported that overexpression of EZH2 (Enhancer of Zeste homolog 2), which is a member of the polycomb group (PcG) of proteins, occurred in a variety of human malignancies including gastric cancer (11,12). The PcG proteins are believed to act as multiprotein complexes that repress target gene expression through modification of chromatin structure. EZH2 is the catalytically active component of polycomb repressive complex 2 (PRC2) and is capable of methylating lysine 9 (H3K9) and lysine 27 (H3K27) of histone H3 (13,14). Moreover, EZH2 was

Table I. Primer sets for MSP.

Primer set	Genome position	Sequence	Product size	Annealing temperature	Cycles
MSP					
BCL2L10-M (S)	-70	AATATATCGGGGGTCGGGGGTC	180	62	35
BCL2L10-M (AS)	+110	AACTCGATACGCTCCCGCAACG			
BCL2L10-U (S)	-70	AATATATTGGGGGTTGGGGGTT	186	59	35
BCL2L10-U (AS)	+116	AACAACAACTCAATACACTCCCA			
P16-M (S)	+167	TTATTAGAGGGTGGGGCGGATCGC	150	65	35
P16-M (AS)	+316	GACCCCGAACCGCGACCGTAA			
P16-U (S)	+167	TTATTAGAGGGTGGGGTGGATTGT	151	60	35
P16-U (AS)	+317	CAACCCCAAACCACAACCATAA			
RUNX3-M (S)	-262	TTACGAGGGGCGGTCGTACGCGGG	220	65	35
RUNX3-M (AS)	-42	AAAACGACCGACGCGAACGCCTCC			
RUNX3-U (S)	-262	TTATGAGGGGTGGTTGTATGTGGG	220	63	
RUNX3-U (AS)	-42	AAAACAACCAACACAAACACCTCC			
hMLH1-M (S)	-675	TATATCGTTCGTAGTATTCGTGT	153	60	35
hMLH1-M (AS)	-523	TCCGACCCGAATAAACCCAA			
hMLH1-U (S)	-721	TTTTGATGTAGATGTTTTATTAGGGTTGT	124	60	35
hMLH1-U (AS)	-598	ACCACCTCATCATAACTACCCACA			

Sense (S) and antisense (AS) primers used for PCR amplification and the size of respective PCR products are shown. Genome position indicates the nucleotide position relative to the transcription start site.

reported to be required for DNA methylation of its target genes through recruitment of DNA methyltransferases (15).

In this study, we clarified the methylation status of BCL2L10 and its expression in gastric cancer tissues and corresponding non-neoplastic mucosae by performing methylation-specific PCR (MSP) and real-time RT-PCR. We also compared it with the methylation status of p16, RUNX3, and hMLH1 genes that have been well documented in gastric cancer. In addition, to determine whether EZH2 influenced the methylation status of these four genes, we examined the association between the methylation status of each gene and the expression level of EZH2.

Materials and methods

Tissue samples and DNA extraction. Gastric cancer tissues and corresponding non-neoplastic mucosae were obtained from 21 patients (male 14; female 7; median age 70 years old; range, 56-85 years) who underwent surgical resection at Chiba University Hospital, Chiba, Japan between 2005 and 2006. All resected specimens were frozen immediately in liquid nitrogen and stored at -80°C until use. Histopathological examination was performed according to the Japanese Classification of Gastric Carcinoma (16). Based on the histological findings, the 21 tumors were classified as 4 well differentiated, 6 moderately differentiated, and 11 poorly differentiated (including signet ring cell and mucinous carcinomas) adenocarcinomas. We microscopically confirmed that the tumor specimens used in this study consisted mainly (>80%) of carcinoma tissue. The status of *H. pylori* infection was also histologically confirmed. All patients had given informed consent for their participation, and the Ethics Committee approved these studies.

Sodium bisulfite DNA sequencing and methylation specific PCR (MSP). Genomic DNAs were extracted using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Using extracted DNA, bisulfite modification of DNA (1 μg) was performed using the CpGenome™ DNA modification kit (Chemicon International Inc., CA, USA) according to the manufacturer's instructions. Modified DNA was purified using a DNA purification kit (Qiagen). To examine the DNA methylation status of the BCL2L10, p16, RUNX3, and hMLH1 genes, we performed MSP. For detection of aberrant methylation of these genes, modified DNA was amplified using AmpliTaq Gold DNA Polymerase (Applied Biosystems), and primers specific for the methylated and unmethylated sequences of each gene (17-19) are shown in Table I. Location of MSP primers for BCL2L10 in its CpG island is shown in Fig. 1. PCR products (5 μl) were run on a 3% agarose gel and visualized by SYBR-Green (FMC, Rockland, ME) staining. CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA) was used as a positive control for methylation.

Semiquantitative RT-PCR and real-time RT-PCR. Total RNA was extracted with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was

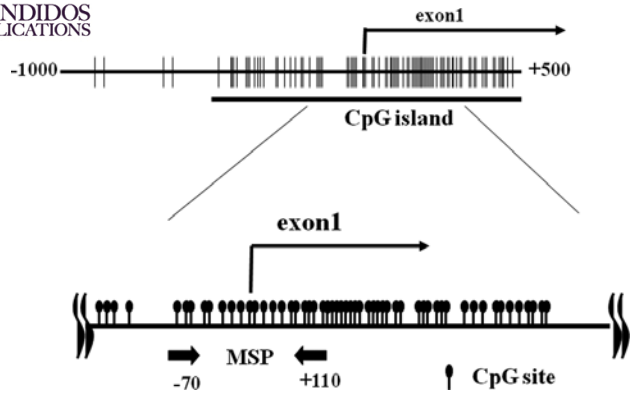

Figure 1. Genomic structure around the 5'CpG island of BCL2L10 from -1000 to +500 with respect to the transcription start site (+1). A vertical bar indicates a CpG site.

synthesized from 1 μg of total RNA by reverse transcription with random oligonucleotide primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and the cDNA was diluted to 200 μl with sterile, ultrapure water. The product (2 μl) was then amplified by PCR using the primer set for EZH2 as previously reported (20). After an initial denaturation at 95˚C for 2 min, PCR was performed in a 25-μl reaction volume for 30 cycles under the following conditions: 95˚C for 30 sec, 62˚C for 30 sec, 72˚C for 60 sec, and an extension at 72˚C for 5 min. PCR products (5μl) were run on a 3% agarose gel and visualized by SYBR-Green staining. RNA samples were also amplified using ß-actin gene primers as control. Real-time PCR was performed for the BCL2L10 gene using Taqman® Gene Expression Assays Inventoried, purchased from Applied Biosystems Japan (Tokyo, Japan). The BCL2L10 gene was amplified on the same plate as the ß-actin reference using the TaqMan Universal PCR Master Mix and the ABI Prism 7000 Sequence Detection Systems (Applied Biosystems), and relative mRNA amounts were determined. Briefly, we normalized each set of samples using the difference in threshold cycles (ΔC_T) between each gene and the ß-actin gene ($\Delta C_{Tsample} = \Delta C_{Tthe\ gene} - \Delta C_{T\beta\text{-}actin}$). cDNA from the cell line, which clearly expressed the mRNA of each gene and the ß-actin gene, was used as the calibration sample ($\Delta C_{Tcalibration}$). Relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta C_{Tsample} - \Delta C_{Tcalibration}$.

Statistical analysis. Differences were analyzed statistically by χ^2 tests and Mann-Whitney U tests. Results were considered statistically significant at $p<0.05$.

Results

Methylation status of BCL2L10, p16, RUNX3, and hMLH1. Promoter methylation of BCL2L10 was detected by MSP in 38% (8 of 21) of gastric cancer tissues and in 24% (5 of 21) of corresponding non-neoplastic mucosae (Fig. 2A and Table II). In 4 cases, BCL2L10 methylation was detected both in gastric cancer tissues and in corresponding non-neoplastic mucosae. CpG island methylation of p16, RUNX3, and hMLH1 was found in both neoplastic and non-neoplastic gastric mucosae, distributed as follows: 43% (9 of 21) and 24% (5 of 21) for p16, 48% (10 of 21) and 19% (4 of 21) for RUNX3, and 10% (2 of 21) and 5% (1 of 21) for hMLH1, respectively (Fig. 2A and Table II). RUNX3 methylation was more frequent in tumors than in non-neoplastic mucosae (p=0.049) (Table III), especially in histologically undiffe-

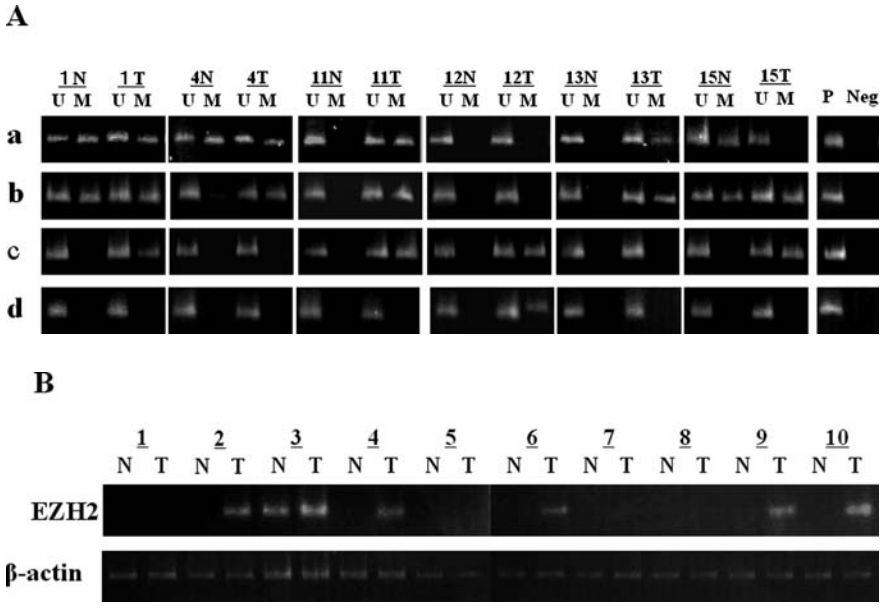

Figure 2. (A) Representative results of methylation-specific PCR (MSP) for BCL2L10 (a), p16 (b), RUNX3 (c), and hMLH1 (d). The numbers of gastric cancer cases are shown above the corresponding lanes. N, non-neoplastic tissue; T, tumor tissue; U and M, MSP analysis specific for unmethylated and methylated sequences, respectively; P, positive control; Neg, negative control (water). (B) Representative results of semiquantitative RT-PCR of EZH2 transcripts in gastric cancer. The numbers of gastric cancer cases are shown above the corresponding lanes. The amplification of ß-actin mRNA was performed to validate the quality of the samples. N, non-neoplastic tissue; T, tumor tissue.

Table II. Clinical characteristics and methylation status of BCL2L10, p16, RUNX3, and hMLH1 and expression of EZH2 in 21 gastric cancer tissues and corresponding non-neoplastic tissues.

	Gastric cancer tissues											Non-neoplastic tissues							
	Sex	Age	Location	Histology	Stage	LNmeta	BCL2L10	p16	RUNX3	hMLH1	EZH2	Atrophy	IM	*H. pylori*	BCL2L10	p16	RUNX3	hMLH2	EZH2
1	F	68	L	Mod	I	-	M	M	M	U	-	+	+	+	M	M	U	U	-
2	M	69	L	Poor	IV	+	U	U	M	U	+	-	-	-	U	U	U	U	-
3	M	62	L	Mod	IV	+	U	U	M	M	+	+	+	-	U	U	M	M	+
4	F	68	L	Well	II	-	M	M	U	U	+	+	-	+	M	U	U	U	-
5	M	73	M	Poor	IIIA	+	M	U	U	U	-	+	+	-	M	M	U	U	-
6	M	85	U	Well	IB	-	U	U	U	U	+	+	+	-	U	U	U	U	-
7	M	76	M	Well	II	+	U	U	U	U	-	-	-	-	U	U	U	U	-
8	F	70	U	Poor	III	-	M	M	M	U	-	+	-	-	M	U	U	U	-
9	M	67	L	Poor	II	+	M	M	U	U	+	-	-	+	U	U	U	U	-
10	M	74	L	Well	IIIA	-	U	U	U	U	+	+	+	+	U	U	U	U	-
11	M	85	M	Poor	IIIA	+	M	M	M	U	-	+	-	-	U	U	U	U	+
12	F	76	L	Poor	IIIB	+	U	U	M	M	-	-	-	+	U	U	U	U	-
13	F	57	U	Poor	IV	+	M	M	M	U	-	-	-	-	U	U	U	U	-
14	M	62	L	Mod	II	+	U	U	U	U	-	+	-	+	U	U	U	U	-
15	M	61	U	Poor	IIIA	+	U	M	U	U	-	+	-	+	M	M	U	U	-
16	M	72	M	Poor	IIIB	+	U	U	M	U	-	+	+	-	U	U	M	U	-
17	M	78	L	Mod	IIIA	+	M	U	U	U	+	+	+	-	U	U	U	U	-
18	M	79	M	Poor	II	+	U	U	M	U	+	+	+	+	U	U	U	U	-
19	F	72	U	Poor	II	-	U	M	M	U	+	+	+	-	U	M	M	U	-
20	M	58	L	Mod	IIIB	+	U	U	U	U	-	-	-	+	U	U	U	U	-
21	F	56	U	Mod	IV	+	U	M	U	U	+	+	+	-	U	M	M	U	-

M, methylated; U, unmethylated; L, lower part of stomach; M, middle part of stomach; U, upper part of stomach; Well, well differentiated; Mod, moderately differentiated; Poor, poorly differentiated.

. Correlation between methylation frequency or n of EZH2 in gastric cancer tissues and those in corresponding non-neoplastic tissues.

	Gastric cancer tissues	Non-neoplastic tissues	p-value
BCL2L10			
Methylated	8	5	0.32
Unmethylated	13	16	
p16			
Methylated	9	5	0.19
Unmethylated	12	16	
RUNX3			
Methylated	10	4	0.0495
Unmethylated	11	17	
hMLH1			
Methylated	2	1	0.55
Unmethylated	19	20	
EZH2 expression			
Present	10	2	0.006
Absent	11	19	

Differences were analyzed statistically by χ^2 test.

rentiated tumors as opposed to differentiated ones (p=0.02). BCL2L10 methylation did not correlate with any clinicopathological characteristics including sex, age, location, histology, stage, or N grade, but did correlate with p16 methylation. p16 methylation was present in 6 of 8 tumors containing methylated BCL2L10 (p=0.02) (Table IV).

Regarding non-neoplastic gastric mucosae, BCL2L10 methylation did not correlate with sex, age, location, presence of intestinal metaplasia (IM), presence of atrophy or *H. pylori* infection, but did correlate with p16 methylation (Table V). RUNX3 methylation was found only in the subjects with IM, not in those without IM (p=0.02). On the other hand, BCL2L10 and p16 methylation were detected in the subjects with atrophy regardless of the presence of IM. No methylation of these genes was detected in those without atrophy. With regard to methylation frequency of each gene, no significant difference was found between subjects either with or without *H. pylori* infection.

Relationship between promoter methylation and mRNA expression of BCL2L10 gene. BCL2L10 expression was analyzed in 21 gastric cancer tissues and corresponding non-neoplastic mucosae by real-time RT-PCR. The expression level of BCL2L10 in tumors was significantly lower than in corresponding non-neoplastic mucosae (p=0.048) (Fig. 3A). Among the 21 tumors, 8 tumors with methylated BCL2L10 showed significantly lower expression of BCL2L10 than the 13 tumors without methylation (p=0.005, Fig. 3B). Among the 21 corresponding non-neoplastic mucosae, 5 subjects with methylated BCL2L10 also showed significantly lower expression of BCL2L10 than the 16 subjects without methylation (p=0.02, Fig. 3C).

Table IV. Correlation of methylation status of BCL2L10 with clinicopathological characteristics and methylation status or expression of p16, RUNX3, hMLH1, and EZH2 in gastric cancer tissues.

Clinicopathological characteristics	Methylation status of BCL2L10		p-value
	M	U	
Sex			
Male (14)	4	10	0.2
Female (7)	4	3	
Age			
≤65 (6)	1	5	0.2
>65 (15)	7	8	
Location			
Upper (6)	2	4	
Middle (5)	2	3	0.96
Lower (10)	4	6	
Histological type			
Differentiated	3	7	0.47
Undifferentiated	5	6	
Stage			
I or II (8)	3	5	0.96
III or IV (13)	5	8	
N stage			
N0 (6)	3	3	0.47
N1, 2, 3 (15)	5	10	
p16 methylation			
Present (9)	6	3	0.02
Absent (12)	2	10	
RUNX3 methylation			
Present (10)	4	6	0.86
Absent (11)	4	7	
hMLH1 methylation			
Present (2)	0	2	0.24
Absent (19)	8	11	
EZH2 expression			
Present (10)	3	7	0.46
Absent (11)	5	6	

Differences were analyzed statistically by χ^2 test. M, methylated; U, unmethylated.

EZH2 expression in gastric cancer and relationship with DNA methylation. EZH2 mRNA abundantly existed in 10

Table V. Correlation of methylation status of BCL2L10 with clinicopathological characteristics and methylation status or expression of p16, RUNX3, hMLH1, and EZH2 in non-neoplastic tissues.

Clinicopathological characteristics	Methylation status of BCL2L10		p-value
	M	U	
Sex			
Male (14)	2	12	0.14
Female (7)	3	4	
Age			
≤65 (6)	1	5	0.63
>65 (15)	3	12	
Location			
Upper (6)	2	4	
Middle (5)	1	4	0.81
Lower (10)	2	8	
IM			
Present (10)	2	8	0.7
Absent (11)	3	8	
Atrophy			
Present (15)	5	10	0.11
Absent (6)	0	6	
H. pylori			
Present (9)	3	6	0.37
Absent (12)	2	10	
p16 methylation			
Present (5)	3	2	0.03
Absent (16)	2	14	
RUNX3 methylation			
Present (4)	0	4	0.21
Absent (17)	5	12	
hMLH1 methylation			
Present (1)	0	1	0.57
Absent (20)	5	15	
EZH2 expression			
Present (2)	0	2	0.41
Absent (19)	5	14	

Differences were analyzed statistically by χ^2 test. M, methylated; U, unmethylated.

of 21 (48%) gastric cancer tissues, whereas it was detectable in 2 of 21 (9.5%) non-neoplastic mucosae, indicating EZH2 expression might be elevated in gastric cancer. We assessed the relationship between the expression pattern of EZH2 and the methylation status of each gene, but there was no apparent correlation in the gastric cancer tissues examined.

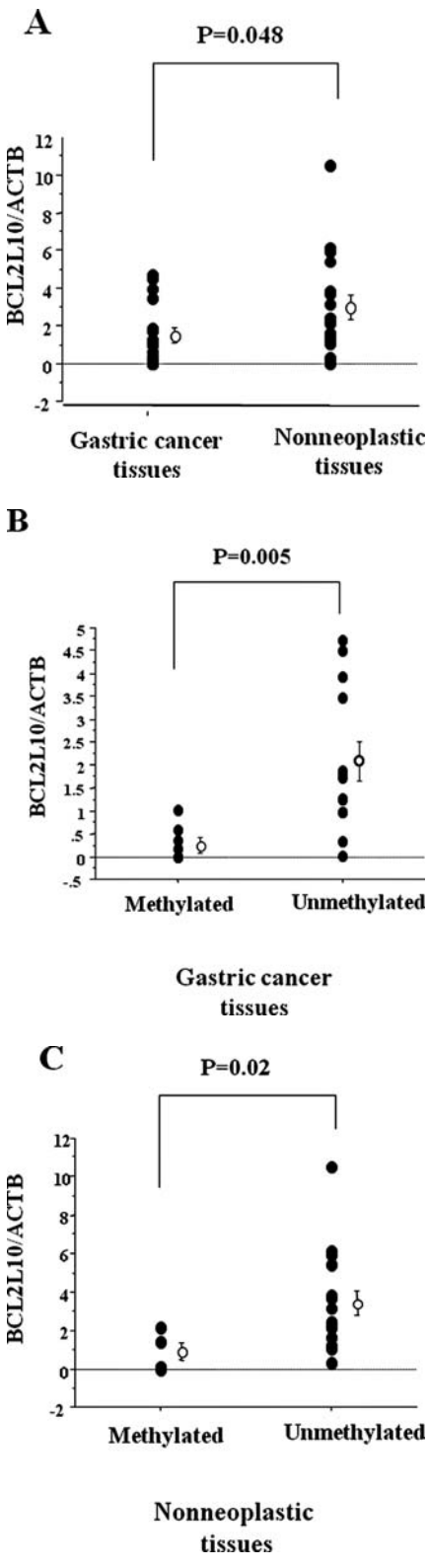

Figure 3. Expression analysis of BCL2L10 by quantitative RT-PCR. (A) BCL2L10 expression was analyzed in 21 gastric carcinoma specimens and corresponding non-neoplastic mucosae. The expression level of BCL2L10 in tumors was significantly lower than in corresponding non-neoplastic mucosae (p=0.048). (B) Among the 21 tumors, 8 tumors with methylated BCL2L10 showed significantly lower expression of BCL2L10 than 13 tumors without methylation (p=0.005). (C) Among the 21 corresponding non-neoplastic mucosae, 5 subjects with methylated BCL2L10 also showed significantly lower expression of BCL2L10 than 16 subjects without methylation (p=0.02). Open circle and vertical bar indicate average and SEM, respectively. Statistical significance was determined using the Mann-Whitney U tests.

In our previous study using cDNA microarray and real-time PCR, five genes were identified to be up-regulated by DAC treatment in gastric cancer cell lines (7). Of these, BCL2L10 was hypermethylated in 4 of 5 gastric cancer cell lines, and its CpG island became demethylated with the restoration of its expression after DAC treatment. We also observed that overexpression of BCL2L10 caused apoptosis and growth-inhibition of gastric cancer cells.

In this study, we used 21 gastric cancer tissues and corresponding non-neoplastic mucosae for investigation of BCL2L10 methylation. As a result, aberrant methylation of BCL2L10 was detected in 38% of gastric cancer and correlated with its low expression, indicating that silencing of BCL2L10 by promoter methylation is a relatively common feature in gastric cancer. According to our previous results, the epigenetic inactivation of BCL2L10 may lead to a loss of apoptosis, and its inactivation by aberrant methylation may be involved in a critical step of gastric cancer development.

MSP analysis demonstrated that methylation of p16, RUNX3, and hMLH1 genes occurred in 43, 48, and 10% of the gastric cancer samples, respectively, consistent with previous reports (21-23). Interestingly, BCL2L10 methylation correlated with p16 methylation status not only in gastric cancer but also in non-neoplastic mucosae, suggesting that a similar mechanism underlies the regulation of methylation status of these genes. Further studies to define the epigenetic regulation of these genes will be necessary.

Regarding non-neoplastic gastric mucosae, aberrant methylation of BCL2L10 was also observed in 24%, while methylation of p16, RUNX3, and hMLH1 was found in 24, 19, and 5%, respectively. These results are consistent with previous reports (21,25). In recent years, methylation of several CpG islands in non-tumorous gastric tissue has been described, especially for the exon 1 region of p16. Jang *et al* showed that p16 methylation was observed in 59% of the non-tumor tissue samples adjacent to gastric cancer and correlated with the presence of grandular atrophy and the grade of chronic inflammation (24). It also has been reported that methylation of p16 and hMLH1 was more frequent in non-neoplastic gastric epithelia of gastric cancer patients than those of non-gastric cancer patients, suggesting these genes may be utilized as predictors of gastric cancer (26). Maekita *et al* reported that methylation levels of specific CpG islands including p16 in non-cancerous gastric mucosa were higher in gastric cancer patients than in age-matched healthy volunteers among *H. pylori*-negative patients using quantitative analysis of methylation (27). In this study, BCL2L10 and p16 methylation were detected in the subjects with atrophy regardless of the presence of IM, whereas no methylation of these genes was detected in the subjects without atrophy. These data suggest that BCL2L10 and p16 methylation might be frequent and early events during the process of gastric carcinogenesis and that BCL2L10 methylation may also serve as a risk marker for the development of gastric cancer. To elucidate the exact prevalence of BCL2L10 methylation in non-neoplastic gastric mucosae, studies of gastric mucosae with non-gastric cancer individuals are needed.

EZH2, a Polycomb group protein, is known to function in histone methylation, thereby regulating gene expression (13,14). Recently, it has been reported that EZH2 was over-expressed in a variety of human malignancies including gastric cancer (11,12). In another report, EZH2 was demonstrated to be required for DNA methylation of its target genes such as the MYT1 gene (15). In mammals, specific DNA sequences that EZH2 recognize as targets have yet to be identified, and target genes of EZH2 are, therefore, still undetermined. In the current study, expression of EZH2 was detected more frequently in tumors (48%) compared with corresponding non-neoplastic mucosae (10%). However, no significant difference was found between expression of EZH2 and methylation frequency of each gene. It is possible that EZH2 might not influence the methylation status of these genes.

H. pylori infection is thought to be an important etiologic risk factor in gastric cancer, and it has been classified as a group of definite carcinogen (28). Some specific CpG islands in *H. pylori*-positive healthy volunteers have been reported to have markedly elevated levels of methylation compared with *H. pylori*-negative counterparts (27). Conversely, it has been also reported that *H. pylori* infection did not correlate with the methylation status of several genes in non-neoplastic gastric mucosa (25,29). In the present study, no correlation was found between methylation of the four genes and presence of *H. pylori* infection. As *H. pylori* infection was evaluated only with histological examination in this study, we can not exclude the possibility of the presence of *H. pylori* in other sites that were not biopsied nor the previous location of *H. pylori*.

In conclusion, our data suggest that silencing of BCL2L10 by aberrant methylation is a common feature in gastric cancer, and its inactivation may be involved in the early steps of gastric carcinogenesis, although further investigation in larger numbers of patients is required.

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